Evaluation of antimicrobial activity of leaf extract of *Canarium Eodontophyllum Miq* (Dabai) in Sarawak

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Abstract

Canarium odontophyllum which is also known as 'Dabai' is one of the popular underutilized fruits in Sarawak. This study was conducted to extract the leaf using methanol, ethanol and acetone as solvent by maceration method with the evaluation of antimicrobial activity using agar well diffusion method. The concentrations of the extracts used are 100 mg/mL, 50 mg/mL, 25 mg/mL and 12.5 mg/mL. The leaf extracts were screened against two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-positive bacteria (*Staphylococcus aureus and Bacillus cereus*) using different solvent extracts. The findings showed that only gram positive bacteria (*s.aureus* and *B.cereus*) was found to be susceptible towards the leaf extracts of methanol, ethanol and acetone compared to gram negative bacteria. *Escherichia coli* and *Pseudomonas aeruginosa* were resistant towards the methanol, ethanol and acetone extracts. These findings will be useful for future study on using *C.odontophyllum* leaves as a new drug in the treatment of bacterial infections.

Keywords: *Canarium odontophyllum*, methanol, ethanol, acetone, maceration, gram positive, gram negative

Abstrak

Canarium odontophyllum juga dikenali sebagai 'Dabai' adalah salah satu pokok yang kurang digunakan sumber-sumbernya di Sarawak. Tujuan kajian ini dijalankan adalah untuk mengekstrak ekstrak dalam tumbuhan menggunakan methanol, etanol dan aseton sebagai bahan pelarut dengan menggunakan kaedah maserasi beserta penilaian aktiviti antibakteria dengan menggunakan kaedah diffusi agar. Kepekatan ekstrak yang digunakan untuk setiap ekstrak adalah 100 mg/ml, 50 mg/ml , 12.5 mg/ml dan 12.5 mg/ml. Kesemua ekstrak ini diperiksa dengan menggunakan dua bakteria negatif (*Escherichia coli* dan *Pseudomonas aeruginosa*) dan dua bakteria positif (*Staphylococcus aureus and Bacillus cereus*). Keputusan kajian menunjukkan bahawa bakteria positif sahaja yang dipengaruhi oleh kesemua ekstrak berbanding bakteria negatif. *Escherichia coli* dan *Pseudomonas aeruginosa* menunjukkan bahawa ianya resistan terhadap metanol, etanol dan juga aseton ekstrak. Kajian ini boleh boleh dijadikan informasi untuk digunakan pada masa akan datang sebagai ubat baru yang boleh mengubati penyakit yang berlaku akibat bakteria.

Kata kunci: Canarium odontophyllum, metanol, etanol, aseton, maserasi, bacteria positif, bacteria negatif

Table of contents

List of tables

List of Figures

List of Abbreviations

B.cereus	*Bacillus cereus*
°C	Degree Celsius
C.odontophyllum	*Canarium odontophyllum*
e.coli	*Escherichia coli*
h	Hour
MHA	Mueller-Hinton Agar
mg/mL	Miligram per mililiter
mL	mililiter
NA	Nutrient agar
OD	Optical density
P.aeruginosa	*Pseudomonas aeruginosa*
S.aureus	*Staphylococcus aureus*
SD	Standard deviation
%	Percent

1.0 Introduction

Canarium odontophyllum is the family of Burseraceae which is also known as 'Dabai' or 'Sibu Olive' commonly found in Sarawak. The different parts of this plant such as the pulp, shell of the seed, the stem bark and the leaf have a different valuable phytochemicals, nutritional and pharmalogical properties (Tan *et al.,* 2016). It had been studied that there is a high level of antioxidant compounds found in the peel of *C. odontophyllum* such as phenolic and flavonoid and it was shown that the leaf and the stem bark of *C. odontophyllum* were shown to have antimicrobial activity and also demonstrated promising anticancer property (Basri *et al.*, 2016). In the study of the cytotoxicity activity of the *C. odontophyllum* leaves extract, the dabai leaves can be chew and drinking dabai tea are considered safe for consumption as chemopreventive supplement against colon cancer as alkaloid is not detected in the leaves of *C. odontophyllum* (Basri *et al.*, 2016).

The increasing usage of antimicrobial activity today has caused certain microorganisms to develop resistance against many antimicrobial agents which lead to the discovery and development of antibiotic agents. The main reason of antibiotic resistance is the misused of the antibiotic. Therefore, the leaves of *C. odontophyllum* is being studied as it has potential antimicrobial activity against medically bacterial and fungal strains and little study conducted on antimicrobial activity in the leaves of *C. odontophyllum.* The leaves of *C.odontophyllum* are much easier to obtain rather than the fruit itself since the fruit seasonal of 'dabai' fruit is from October to January and it is also can be obtained in abundance. Since the bacteria genomes shown a drastically changes and resistance towards the antibiotics, these contribute to the finding of the natural drug from this plant.

Canarium odontophyllum also can be categorized as the indigenous tropical fruit that had potential for commercial development in Malaysia (Khoo *et al.*, 2016). It is being used in medicinal studies but these fruits is being classified as one of the popular underexploited and underutilized fruits due to the lack of promotion, minimal planting area with their economic potential not being fully explored (Basri *et al.*, 2014). Therefore, there are many variations in health benefits and bioactive phytochemicals can be found and studied in these plants for further research which can turn them into one of the plants that highly potent to be commercialized (Khoo *et al.*, 2016)

The objectives of this research are to extract antibacterial properties of *Canarium odontophyllum* leaves extract using methanol, ethanol and acetone extraction method. Other than that, this research is being studied to evaluate antimicrobial activity from the crude extract against selected gram positive and gram negative bacteria using agar well diffusion method.

2.0 Literature Review

2.1 *Canarium* species

There are 75 species of genus *Canarium* trees that can be found in the tropical rainforests of tropical Asia, Africa and the pacific and the *Canarium* species have been used to treat a broad array of illness traditionally (Mogana *et al.*, 2011). The examples of genus *Canarium* are *Canarium album*, *Canarium littorale, Canarium parvum, Canarium perlisanum, Canarium patentinervium* and others. It is reported that the extract and pure compounds derive from the genus *Canarium* have a variety of pharmacological activities such as antibacterial, antifungal, antitumor, hepatoprotective, antioxidant and anti-diabetic (Mogana *et al.*, 2011). One of the *Canarium* species that been proven to have the high antimicrobial properties is *Canarium schweinfurthii* which is the African olive (Obame *et al.*, 2007). The examples of the traditional medicine used in *Canarium* species are the dried fruit of Chinese olive or *Canarium album* can be used to treat viral infections, inflammation, poisoning and also detoxification and it have been used for treatments of snake bites, dysentery, enteritis and also swellfish (Mogana *et al.*, 2011).

2.2 *Canarium odontophyllum Miq.*

Canarium odontophyllum Miq which is commonly known as 'dabai' is one of the popular underutilised fruits of Sarawak, Malaysia. The fruit seasonal of dabai fruit is from October to January and the storage period of dabai is about two days after harvest (Xuan *et al.*, 2016). It is usually found naturally along river banks in Sibu, Sarikei, Kapit and Limbang divisions. Dabai fruit are rich in nutrients such as carbohydrates, protein, sodium, fat, calcium and iron and it being reported that this fruit have many phytochemicals from the different parts of the fruit. It is a good source of unsaturated fatty acids and there are some products that have been developed from this fruits for local markets such as chips,

sauces, chips, pickles and soap. Therefore, the other part of these plants might have valuable properties that can be used to be in the market such as the leaves part of this plant.

Figure 2.1: *Canarium odontophyllum* leaves

2.3 Medicinal properties of plants

Plant is one of the sources to produce antimicrobial agent or product for the inhibition of the bacterial and fungal growth. Medicinal plants consider as a rich resources of ingredients which can be used in drug development and synthesis (Hassan, 2012). There are various groups of compounds in the plant which are effective as an antimicrobial compound which are flavones, flavonoids, flavonols, coumarins, phenols, phenolics, alkanoids, quinines, lectin and polypeptides. These molecules are usually derived from the secondary metabolism of plants and were used to protect it against predation such as microorganism, herbivorous and insects (Basri *et al.*, 2014).

There are three large molecules families of antibacterial secondary metabolites which are phenolic, alkaloid and terpene. The use of this type of medicine as a safe remedy for diseases of both microbial and non-microbial origin has been supported by World Health Organization (Basri *et al.,* 2014). Almost all the parts of plants were tested including the pulp, leaf, shell of the seed and the stem bark. The leaf and the stem bark of *C. Odontophyllum* were shown to have antimicrobial activity and also demonstrated promising anticancer property.

About 70% of the world's population relies on plants for their primary healthcare, 35,000 to 70,000 species been used as medicaments (Mamedov, 2012)

2.4 Leaves extraction method

Crude extraction method is the method where the plant extract mixed with various solvents for example methanol, ethanol, acetone, hexane and water. According to Sasidharan (2011), in the analysis of the plants, extraction is the first crucial step as it important to extract the components from the plants for the characterization and separation. The function of crude extraction is to inhibit the pathogenic bacteria and fungi (Hussain and Ananthan, 2009). The organic solvents such as methanol and acetone are commonly being used because plant compounds are saturated organic compound (Khoddami *et al.*, 2013). The different of extracting using various solvent is that they convey different level of polarity and the percentage yield of extraction during phytochemical analysis is different (Basri *et al.*, 2014) Maceration, infusion, digestion, percolation, hot continuous extraction (Soxhlet), aqueous extraction by fermentation and decoction are an example of extraction method. Maceration techniques is the process that soaking plant materials in the form of coarse or powdered together with a solvent in a stoppered container in order to soften and break the plant's cell wall (Azwanida, 2015). Hot continuous extraction which is also known as Soxhlet method is the technique where the powdered plant sample is place in the "thimble" or specialize porous bag soaked into the solvent in the soxhlet equipment and heated for 5 h or until the solvent do not leave residue when evaporated. Maceration method is being used in this experiment since it is the simplest method and it does not expose to hazardous and flammable liquid organic solvents as Soxhlet do although it is time consuming.

2.5 Agar well diffusion method

Agar well diffusion method one of the method that is widely in the antimicrobial activity. It is much similar with the disc diffusion method whereas after the surface of the agar is seeded with the test bacteria, 6 to 8 mm are punched to make the well (Balouiri *et al.,* 2015). Then, the extract concentrations with the desired concentrations being introduce into the well. Disc diffusion method also is one of the commonly used method in antimicrobial study due to its simplicity and it is cost effective (Scorzoni *et al.,* 2007). It is also easy to handle and no need to use any specialized equipment and only required a small amount of volumes to be use. In this study, agar well diffusion is being used as by using this method, the sample can be loaded more into the well compared to the disc diffusion method.

2.6 Gram positive and Gram negative

Staphylococcus aureus is Gram positive bacteria and it is one of the major pathogens of increasing importance due to the rise in antibiotic resistance (Lowy, 1998). *S. aureus* give high resistance against antibiotics and they destroy neutrophils. *Bacillus cereus* is a Gram positive bacteria and have ability to cause foodborne diseases (Tajkarimi, 2007). It is usually associated with the food poisoning which can cause fatal non-gastrointestinal-tract infections. *Escherichia coli* is a Gram negative bacteria and most of strains of e.*coli* are harmless but some species of *e.coli* can make people sick as they possessed virulence gene (Doyle, 1989). *Pseudomonas aeruginosa* is a Gram negative bacteria and one of the leading nosocomial pathogens worldwide (Strateva and Yordanov, 2009). It represents a phenomenon of antibiotic resistance (Haynes, 1951).

3.0 Materials and Methods

3.1 Materials

3.1.1 *Canarium odontophyllum Miq* leaves

The fresh leaves of *Canarium odontophyllum Miq* obtained in areas of Kuching, Sarawak.

3.1.2 Organic solvents

The organic solvents used for the extraction were methanol, ethanol and acetone.

3.1.3 Microorganisms

The bacteria used were two Gram positives bacteria which are *Bacillus cereus* and *Staphylococcus aureus* and two Gram negatives bacteria which were *Pseudomonas aeruginosa* and *Escherichia coli*.

3.1.4 Agar media and broth

The agar media used were Nutrient agar for the culture of test bacteria and Mueller Hinton Agar (MHA) in the screening of antimicrobial activity. Mueller Hinton Broth used in culturing the single colony of tested bacteria.

3.2 Methods

3.2.1 Preparation of Leaves

The leaves of *canarium odontophyllum* being wash for several times using distilled water to remove the dirt and impurities on the surface of the leaves in order to increase the accuracy of the experiment. Then, the leaves were air dried for a week. Next, the leaves cut into smaller pieces before grounded into powdered form using the electric blend (Narutron).

3.2.2 Preparation of extracts

Powdered leaves 10 g of *Canarium odontophyllum* leaves being extracted by three organic solvents which are methanol, ethanol and acetone with different ratio 1:30 (w/v, 10 g of powder in 300 ml solvent). In methanol extraction, dried materials immersed in the methanol by maceration method for 3 days as being describe by Basri *et al.,* (2014) while being shake using the rotarory shaker at 120 rpm (Maher *et al.,* 2012). Then, the mixture was filtered using Advantec filter paper (125 mm) to separate the residue and the solvent. The solvent then was evaporated using the rotarory evaporator as being described by Basri *et al.,* (2014) at 35-37 ° C for 30 to 45 minutes. After that, the crude extracts were taken out and weight. The similar procedure was repeated for ethanol and acetone extract.

3.2.3 Preparation of extraction solution

The stock solution of 100mg/ml was prepared by dissolving the extract in blank 7.8 % DMSO solution. Two-fold dilution method was used to prepare the concentrations of the crude extracts. The stock solution reduced into 100 mg/ml, 50 mg/ml, 25mg/ml and 12.5mg/ml.

3.2.4 Preparation of Microorganisms

All the bacterial strains of *Staphylococcus aureus, Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli* were grown on nutrient agar (NA) and incubated for 24 hours at 37 °C as being described by (Basri *et al.,* 2014). One single colony from each bacteria on NA being taken and inoculated the bacteria on Mueller Hinton Broth (21 g of MHB powdered in 1000 ml of distilled water to make the broth). The plates then being incubated for 24 hours at 37 °C before used according to Umachigi *et al.,* (2007). Then, the inoculums size of each of the strains being standardized by using the spectrophotometer. The turbidity was then adjusted in order to match 0.5 McFarland standard solution at 625 nm which is corresponding to 2.4 x 10^8 c.f.u./ml according to Roy *et al.,* (2010).

3.2.5 Screening for Antibacterial Activity

The screening of antibacterial activity of *C. odontophyllum* leaves were carried out by using agar well diffusion method as described by Eloff (1999). The test bacteria spread on the surface of Mueller Hinton agar (MHA) by using sterile cotton swab. The plate that already being seeded with the bacteria is being punched to make well of 6 mm of diameter by using a sterile Pasteur pipette (Basri *et al.,* (2014). The total of six well are punched off on each plate for positive and negative control and four different concentrations of each extracts. The extract concentration of 50 µl of 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml, 7.8 % blank DMSO as negative control and 25 µl of Gentamycin as positive control were pipetted into the well on the agar that had been seeded with tested microorganisms. Then, the plate incubated for 24 hr at 37°C before the diameter of inhibitions zone surrounding the well were measured to determine the antibacterial activity. Each experiment were done in triplicate to calculate the mean value ± SD value.

4.0 Results

The antibacterial activity of the methanol, ethanol and acetone extracts against gram positive and gram negatives bacteria being measured by observing the inhibition zone. The different concentration of extracts of 100 mg/ml, 50 mg/ml, 25 mg/ml and 12.5 mg/ml on different test bacteria are being measures. The positive and negative control used in this study are 1 mg/ml of Gentamycin and 7.8% Blank DMSO. The results are being describes in the Table 4.1, Table 4.2 and Table 4.3.

4.1 Methanol extraction

Results showed the summary of the antibacterial activity of *C.odontophyllum* leaf of methanol extracts against four test bacteria in triplicates. The 100 mg/ml of *Bacillus cereus* show the greatest inhibition zone of 11.66 ± 2.94, followed by *Staphylococcus aureus* 10.66 ± 0.47. For the 50 mg/ml concentration, *Bacillus cereus* and *Staphylococcus aureus* show the inhibition zone of 9.66 ± 0.64 and 10.66 ± 2.94, while the 25 mg/ml concentration, *Bacillus cereus* and *Staphylococcus aureus* show the inhibition zone of 9.33 ± 2.16 and 8.66 ± 2.16 respectively. The lowest concentration of 12.5 mg/ml, the inhibition zone for both *Bacillus cereus* and *Staphylococcus aureus* are 8.66 ± 0.82 and 7.66 ± 0.82 which showing the lowest inhibition among the other concentrations. In the gram negative of both *Escherichia coli* and *Pseudomonas aeruginosa,* there are no inhibition zone in all of each concentrations because the bacteria are unsusceptible and probably because the methanol extracts did not have antibacterial properties towards gram negative bacteria. The positive control of *Bacillus cereus, Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa* are 29.33 ± 3.56, 25.33 ± 1.49, 20.00 ± 0.00 and 19.00 ± 0.00. The positive control also shows the greatest inhibition zone in *Bacillus cereus* followed by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*. This methanol

extraction showed that both gram positive showed a steady increase of antimicrobial activity with the increase of the extract concentration. The results are shown in the Table 4.1.

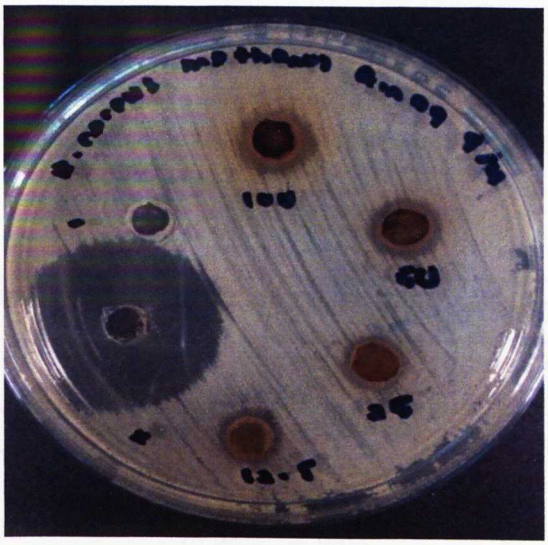
Table 4.1: Mean diameter of inhibition zone of methanol extracts towards *staphylococcus aureus, Escherichia coli, Bacillus cereus and Pseudomonas aeruginos*

Extracts	Extracts concentration (mg/ml)	Mean diameter of inhibition zone (mm)			
		S.aureus	***E.coli***	***B.cereus***	***P.aeruginosa***
Methanol	100	10.66 ± 0.47	-	11.66 ± 2.94	-
	50	9.66 ± 0.64	-	10.66 ± 2.94	-
	25	8.66 ± 0.82	-	9.33 ± 2.16	-
	12.5	7.66 ± 0.82	-	8.66 ± 2.16	-
Positive control	Gentamycin	25.33 ± 1.49	20.00 ± 0.00	29.33 ± 3.56	19.00 ± 0.00
Negative control	7.8% of DMSO	-	-	-	-

Results expressed in mean, ± = standard deviation, (-) = no inhibition

Figure 4.1: Zone of inhibition of methanol extracts towards a) *Staphylococcus aureus,* b) *Bacillus cereus,* c) *Escherichia coli* and d) *Pseudomonas aeruginosa*

a) *Staphylococcus aureus* (gram +)



b) *Bacillus cereus* (gram +)

c) *Escherichia coli* (gram -)

d) *Pseudomonas aeruginosa* (gram -)